European Journal of Epidemiology (2005) 20: 833–838
DOI 10.1007/s10654-005-2148-7

Collecting saliva by mail for genetic and cotinine analyses in participants recruited through the internet

Jean-François Etter[1], Elisabeth Neidhart[2,3], Sonia Bertrand[3], Alain Malafosse[2] & Daniel Bertrand[3]

[1]*Faculty of Medicine, Institute of Social and Preventive Medicine, University of Geneva, Geneva, Switzerland;* [2]*Department of Psychiatry, Geneva University Hospitals, Geneva, Switzerland;* [3]*Faculty of Medicine, Department of Physiology, University of Geneva, Geneva, Switzerland*

Accepted in revised form 15 August 2005

Abstract. The authors assessed whether collection by mail of saliva and buccal cells for genetic analysis was feasible in participants recruited through the Internet. In 2003, 14,773 visitors of a smoking cessation website were invited by e-mail to take part in the study. Salivettes (plastic vials containing a cotton roll) were mailed to participants, for collection of saliva and buccal cells. Because of limited resources, the authors stopped recruitment when 392 participants (3% of 14,733) were registered. They received 315 saliva samples back (80% of 392). Salivary cotinine was analyzed in 145 daily smokers. Cotinine concentration could be assessed in 141 samples (97%) (range 0.7–899 ng/ml, median 260 ng/ml). DNA extraction was achieved in all the 285 samples in which it was attempted. Quality of DNA was assessed by optical density measurements and by polymerase chain reaction amplification of a gene coding for the α-4 nicotinic receptor, with the detection of a known polymorphism. Successful results were obtained in 235 samples (82% of 285). Thus collecting saliva by mail for cotinine and DNA analysis in participants recruited through the internet produced samples of good quality at a reasonable cost. This approach should be valuable for genetic epidemiology and pharmacogenetic research.

Key words: Cotinine, Epidemiology, Internet, Nicotine Dependence, Nicotinic Receptors, Smoking

Introduction

The most common source of deoxyribonucleic acid (DNA) for both clinical and research purposes is from blood lymphocytes. But the collection of blood samples requires venipuncture, may cause some pain and phobia, carries some risk and must be performed by trained personnel. Some patients dislike venipuncture and may not participate in studies that require it. Exfoliated buccal epithelial cells and other cells found in saliva are an appropriate alternative source of genomic DNA [1–4]. These cells can be obtained using non-invasive, self-administered and inexpensive procedures, such as buccal swabs (cytobrushes) and mouthwash [2–4]. DNA from buccal cells is stable when the samples are stored at room temperature during several days [5], thus it is possible to collect the samples by mail.

Because of the large number of participants required in genetic epidemiology or pharmacogenetic studies, the cost of data collection can be prohibitive when the data include questionnaires, cells for DNA analysis and saliva for the analysis of, for instance, cotinine (a metabolite of nicotine), hormones, HIV antibodies or the concentration of medications and their metabolites [6, 7]. The internet can be used to collect on-line questionnaire data from large samples at a low cost. Internet participants have already been asked to provide saliva samples for cotinine analysis [8], but they have seldom been asked to send samples by mail for DNA analyses in epidemiologic studies. Thus little is known about the number of internet participants who will provide cells for DNA analyses, in a context where participants have no in-person contact with the researchers and may know little about them and about the institution where the research is conducted.

The aim of this study was to assess the feasibility of collecting saliva samples by mail for DNA and cotinine analysis, from participants recruited over the internet, for a pilot study of associations between smoking behavior and the genes that code for nicotinic receptors.

Methods

This was a pilot study, aimed at testing the data collection and laboratory methods for a future and larger study of associations between smoking and the polymorphisms of genes that code for nicotinic acetylcholine receptors. In September 2003, we sent

by e-mail an invitation to take part in the study to 14,773 current, former and never smokers who had previously visited a smoking cessation website (www.stop-tabac.ch), had indicated their e-mail address and agreed to receive information by e-mail from this website [9]. A link to the study homepage was also posted on the website of the Faculty of Medicine of the University of Geneva. Potential participants first read an information page about the study and the data protection procedures. This document indicated that the study had been approved by an ethics committee, and that participants were required to provide a saliva sample for the analysis of cotinine and of the genes that code for nicotinic receptors (briefly described as receptors in the brain that may be involved in tobacco dependence). Then, participants completed a consent form on the screen, indicated their name and address and completed an on-line questionnaire. Eligibility criteria included age $\geqslant 18$ and residence in Switzerland. We sent a salivette (Sarstedt, Nümbrecht, Germany) by mail to participants. A salivette is a plastic vial that contains a small cotton roll, similar to cotton rolls used by dentists. Participants were instructed to chew the cotton roll during 30–45 sec before replacing it in the plastic vial. They were told to collect the sample at least 30 min after eating or drinking, and before brushing their teeth. Participants who received a salivette were invited to sign a second consent form on paper and to send it back to us with the saliva sample.

Questionnaire content

The questionnaire (in French) included 160 questions and is available at http://www.stop-tabac.ch/gen/tous-r-09.htm. The questionnaire covered age and sex, education and income, smoking behavior, motivation to quit smoking, dependence on cigarettes (FTND and CDS-12 scales) [8, 10], the CES-D depression scale [11], the Neuroticism scale from Eysenck's Personality Questionnaire (EPQ-R) [12] and the Novelty Seeking scale from Cloninger's Temperament and Character Inventory (TCI) [13].

Ethics and data management procedures

The study was approved by the Geneva ethics committee for research in public health, and the data collection and data management procedures were approved by the Geneva State Council, according to Swiss laws on data protection.

Cotinine analysis

Upon reception, salivettes were stored in the refrigerator at 4 °C. Liquid contained in the salivettes was extracted by a gentle centrifugation (500 g/2 min) and cotton rolls were removed. After centrifugation, saliva samples were frozen at −20 °C until they were shipped on dry ice by express mail to ABS Laboratories, London (www.abslabs.com), for cotinine analysis. Cotinine was analyzed by gas–liquid chromatography [14]. We analyzed cotinine in daily smokers only (n = 145), identified by their answers to the questionnaire.

DNA analysis

DNA extraction from the cotton rolls was done after centrifugation, using the DNA extraction kit from Promega (Zürich, Switzerland). Briefly, cotton rolls were imbibed with 1 ml Lyse:Nuclei solution (Promega) with addition of 20 μl proteinase K (20 mg/ml). Tubes were incubated overnight at 55 °C to detach cells from the cotton rolls and lysate remaining cells. The cotton rolls were then pressed in a syringe to extract the liquid and 50 μl MagneSil Blue was added to adsorb the proteins (Promega). Following separation of the magnetic bids retaining the proteins, Lyse:SV lysis buffer (Promega) was added at equal volume. DNA was then extracted by vacuum filtration on small column (96 wells format, Promega). DNA was washed three times with ethanol solution (Promega), suspended in distilled water and transferred by vacuum in a tube (96-well plates). All samples were adjusted to a final volume of 120 μl. DNA quality was assessed by optical measurements with detection of the 260/280 nm ratio.

To further assess DNA quality and to maintain compatibility with the 96-well plate format, polymerase chain reactions (PCR) were carried out using standard protocols in the first 285 sample received only. Forward primer was GGCGAGTGGGTCATC GTGG and reverse primer was GATGACCAGTGA GGTGGACG, delimiting a short segment of exon 5 of the gene coding for the nicotinic cholinergic receptor α-4, used to amplify an appropriate amount of DNA. Annealing temperature was 64 °C. Agarose electrophoresis gels were performed and clean DNA bands were observed in 82% of the cases (235/285). No correct DNA band was observed in the remaining 50 cases. Following PCR amplification, enzymatic digestion was made with Cfo-I at 37 °C for 9 h.

Statistical analyses

We used chi-square tests to compare proportions, *t*-tests to compare means and Mann–Whitney *U*-tests to compare medians.

Results

Participation

Because of limited resources for laboratory analyses, we interrupted data collection after 18 days, when 392 complete records were stored. We sent a salivette

to these 392 participants, but 5 envelopes came back because of invalid postal addresses. We received 315 saliva samples (81% of 387 participants with a valid address). The follow-up on-line survey conducted after 1 month was answered by 346 people (88% of 392). Characteristics of participants are described in Table 1.

Vials sent back

Of the 315 vials received, two were broken, probably by sorting machines in the postal offices, but remained suitable for DNA extraction. In three cases, however, the cotton rolls had not been used.

Cotinine analysis

Three saliva samples (2% of 145 samples sent out for analysis) had insufficient volume for cotinine analysis, and one sample was swamped by some other compound, which made it unusable for cotinine analysis. All the remaining 141 samples (97% of 145) had a detectable level of cotinine (range 0.7–899 ng/ml, quartiles 153, 260 and 380 ng/ml). Five samples (3%) had cotinine levels below 10 ng/ml, a conventional threshold used to distinguish smokers from non-smokers [15]. Four of these five participants took part in the 1-month follow-up survey and indicated that they had quit smoking at follow-up. These four people reported that they had smoked during respectively 0, 2, 4 and 8 days during the 30 days before the 1-month follow-up survey, and three of them said they had not smoked any cigarette in the 7 days prior to follow-up.

Extraction of DNA and genetic analysis

Significant amounts of DNA were extracted from 312 out of the 315 tubes received (99%). Optical measurements performed at two wavelengths (see methods) were used as a first assessment of the DNA quality. In the 312 samples, mean DNA concentration was 69.21 ± 44.24 ng/μl. Taking into account the final volume of 120 μl this corresponds to roughly 8.2 μg DNA, which is sufficient to perform roughly 160 PCR amplifications. For practical reasons and use of 96-well plate format, PCR were attempted in the first 285 samples available only. Adequate amplification was obtained in 235 cases (82% of 285) and DNA was observed in agarose gels and suitable polymorphism analysis could be carried out.

Cost analysis

The collecting cost per vial was approximately CHF 3.00 (USD 2.50) (salivette CHF 0.35 per piece, post stamps CHF 1.70 per sample, letters and envelopes CHF 1.00 per participant). Cotinine analysis was

Table 1. Among people who answered a questionnaire on the internet, comparison of those who returned a saliva vial for cotinine and DNA analysis with those who did not return the vial

	Returned a saliva sample N = 315	Did not return a saliva sample N = 72	*p*-value
Age	40	39	0.37
Men (%)	44	54	0.12
Years of education	15	15	0.75
Household income (% 'above average')	45	38	0.70
Depression, average CES-D score	11.5	15.1	0.005
Depression, % with CES-D score $\geqslant 16$	27.2	46.8	0.002
Ever diagnosed with depression (%)	33	35	0.95
Neuroticism, from EPQ-R (mean)	11.1	12.0	0.24
Novelty Seeking, from TCI (mean)	21.3	22.4	0.23
Took part in the 1-month follow-up (%)	94	65	< 0.001
Smoking status (%)			0.002
Never smokers	5	0	
Ex-smokers	42	24	
Occasional smokers	3	5	
Daily smokers	49	74	
Among daily smokers			
Nicotine dependence (FTND, mean)	4.6	5.1	0.20
Cigarette dependence (CDS-12, mean)	45.5	45.6	0.97
Cigarettes per day (mean)	23	25	0.37
Minutes to first cig. of the day (mean)	54	29	0.053
Made a quit attempt in past year (%)	51	34	0.045
Intends to quit in next 6 months (%)	79	96	0.019
Among ex-smokers			
Months since quit smoking (median)	16	10	0.34

UK£ 8 per sample (USD 15), and DNA extraction and analysis was roughly CHF 10 (USD 8) per sample. Thus, the total cost of collecting and analyzing cotinine and DNA (one polymorphism only) was approximately USD 26 per sample, not including the labor cost.

Discussion

It was possible to recruit rapidly a substantial number of participants on a smoking cessation website for a genetic epidemiology study. Most participants who received a vial (81%) returned a saliva sample for cotinine and DNA analysis. In almost all (98%) samples returned by daily smokers, the quantity of saliva was sufficient for cotinine analysis, and all the samples with enough volume had a detectable level of cotinine. DNA analysis by PCR was possible in a majority of samples (82%). Thus the quality of the saliva and cell samples collected with this method was good and suitable for analysis.

DNA

Salivettes have seldom, if ever, been used to collect DNA by mail for epidemiological studies. This study confirms that this procedure is feasible, simple, inexpensive and acceptable by study participants. This non-invasive technique poses less risk than venipuncture and may be used outside a medical setting, since it requires no supervision of patients by qualified personnel and no training of participants. While several methods exist for the DNA extraction, we selected a method that required a minimal workload and that is easily amenable for automated use. Results presented herein illustrate that this method provides good DNA quality while also allowing the possibility of saliva analysis.

With PCR techniques, a very small quantity of DNA is sufficient for analysis. This study showed that salivettes collected by mail produced DNA in sufficient quantity and good quality. Several techniques can be used to collect DNA by mail, including mouthwash, mouth brushes, or hair roots [2–4]. Compared with these techniques, salivettes have the advantage of needing only one sample for two purposes: analyses of DNA and saliva. The validity of using buccal cells for DNA analysis has been demonstrated previously [1–4], and research showed than genomic DNA from buccal cells collected by mouthwash is stable for prolonged periods at room temperature, allowing collection of the samples by mail [5]. Thus this approach should be suitable for large population studies, in genetic epidemiology or pharmacogenetic research, in which samples are collected by the participants themselves, sent by mail and stored for weeks or months before cotinine or DNA extraction and analysis. Saliva can also be used to test hormones [6], HIV antibodies [7], or the concentration of medications and their metabolites [16]. Thus this procedure can be used in pharmacogenetic studies, i.e. studies that seek to determine the genetic basis for drug response.

Correct PCR amplication and polymorphism analysis could be carried out in 82% of the DNA samples in which it was attempted. This indicates that good quality DNA was obtained in most cases and that this method is suitable for polymorphism studies. However, a higher percentage of valid samples could probably be obtained by giving more detailed or graphic instructions to participants and by personal follow-up in those who do not provide a valid sample. An alternative method of DNA collection by mail using a mouthwash was recently published [3]. While mouthwash and other methods may yield higher amount of DNA, the quality of the extraction remains the determining criterion for the usability of the samples. In this respect, it should be noted that the salivettes method described here yielded high molecular weight DNA in most of the extracts, and that the percentage of responses was substantially higher in the present study, suggesting an advantage of the use of salivettes over mouthwash.

Cost

Collecting salivettes by mail and analyzing saliva cotinine and DNA could be achieved at a reasonable cost. Thus this approach should be valuable for epidemiological studies that typically require large numbers of participants.

Limitations of this approach

Because of budget limitations, we interrupted data collection before recruiting the maximum possible number of participants, but there was nevertheless a very low response rate, of less than 3% of those who were invited by e-mail to take part in the study. We have no information on the predictors of participation among people who received the e-mail. Among those who answered the questionnaire and received the salivette, current smokers and depressed people were less likely to return a saliva sample, which may limit the generalizability of the results.

The study was conducted in a self-selected sample of users of a smoking cessation website and as expected, the sample included very few never smokers. In the future, internet recruitment should be conducted on various websites to increase the diversity of study samples. Compared with representative samples of smokers in Europe or the US, smokers in this study were more motivated to quit smoking (80% vs. 25–50% intended to quit in the next

6 months) [17, 18]. In a previous study, we compared smokers self-recruited on the same website with smokers who took part in a mail survey [19]. We found that even though the *distribution* of smoking-related variables was different in the two samples, the strength of *associations* between variables was similar in smokers recruited on the internet or by mail. Since genetic epidemiology studies focus on associations between variables, internet data collection should produce generalizable results. However, analyses of the genetic determinants of smoking in representative population samples are warranted.

Previous research showed that about 20% of the general population have CES-D scores $\geqslant 16$, substantially less than the 27% observed in this sample. This probably reflects the high proportion of smokers in this sample, or that depressed people, being less confident in their ability to quit smoking [20], were more likely to visit the website, looking for support. Neuroticism scores were close to normative values [21], but Novelty Seeking scores (mean = 21.3) were higher than normative data from France (mean = 16.4) and the US (mean = 19.2) [13, 22]. Previous research showed that Novelty Seeking scores are higher in current smokers than in never smokers [23, 24]. Thus the high Novelty Seeking scores may simply reflect the high proportion of smokers in our sample. This result may also reflect that Novelty Seekers are more likely to use the internet and to be willing to participate in research.

Another concern is that we could not control whether the saliva samples were provided by the same persons who answered the questionnaire. However, participants indicated their name, age and sex in the internet survey and in the informed consent forms on paper sent back with the saliva samples. All names and data on age and sex corresponded to the same person for each record, which provides some evidence that this data collection method is reliable [25].

Finally, this data collection method produced enough DNA for classical candidate gene studies, but not enough for a whole genome association study.

We conclude that collecting a single saliva sample by mail for the analysis of both cotinine and DNA in participants recruited through the internet was feasible and produced samples of sufficient quantity and good quality, at a reasonable cost. This approach should be valuable for genetic epidemiology and pharmacogenetic research.

Acknowledgements

This study was supported by grants from the Swiss National Science Foundation to J.-F. Etter (3233-054994.98 and 3200-055141.98), to A. Malafosse (32-10216) and to D. Bertrand (3100A0-101787). Vincent Baujard, from the HON Foundation (www.hon.ch) developed the software for data collection.

References

1. Garcia-Closas M, Egan KM, Abruzzo J, et al. Collection of genomic DNA from adults in epidemiological studies by buccal cytobrush and mouthwash. Cancer Epidemiol Biomarkers Prev 2001; 10: 687–696.
2. Lum A, Le Marchand L. A simple mouthwash method for obtaining genomic DNA in molecular epidemiological studies. Cancer Epidemiol Biomarkers Prev 1998; 7: 719–724.
3. Bauer J, Rezaishiraz H, Head K, et al. Obtaining DNA from a geographically dispersed cohort of current and former smokers: Use of mail-based mouthwash collection and monetary incentives. Nicotine Tob Res 2004; 6: 439–446.
4. Witso E, Stene LC, Paltiel L, et al. DNA extraction and HLA genotyping using mailed mouth brushes from children. Pediatr Diabetes 2002; 3: 89–94.
5. Andrisin TE, Humma LM, Johnson JA. Collection of genomic DNA by the noninvasive mouthwash method for use in pharmacogenetic studies. Pharmacotherapy 2002; 22: 954–960.
6. Dabbs JM Jr. Salivary testosterone measurements: Collecting, storing, and mailing saliva samples. Physiol Behav 1991; 49: 815–817.
7. Van den Akker R, van den Hoek JA, van den Akker WM, et al. Detection of HIV antibodies in saliva as a tool for epidemiological studies. AIDS 1992; 6: 953–957.
8. Etter JF, Le Houezec J, Perneger TV. A self-administered questionnaire to measure dependence on cigarettes: The cigarette dependence scale. Neuropsychopharmacology 2003; 28: 359–370.
9. Bock BC, Graham AL, Sciamanna CN, et al. Smoking cessation treatment on the Internet: content, quality and usability. Nicotine Tob Res 2004; 6: 207–219.
10. Heatherton TF, Kozlowski LT, Frecker RC, et al. The Fagerström Test for Nicotine Dependence: A revision of the Fagerström Tolerance Questionnaire. Br J Addict 1991; 86: 1119–1127.
11. Radloff LS. The CES-D scale: A self-report depression scale for research in the general population. Appl Psychol Measure 1977; 1: 385–401.
12. Eysenck SBG, Mc Gurk BJ. Impulsiveness and venturesomeness in a detention center population. Psychol Rep 1980; 47: 1299–1306.
13. Cloninger CR, Svrakic DM, Przybeck TR. A psychobiological model of temperament and character. Arch Gen Psychiatry 1993; 50: 975–999.
14. Feyerabend C, Russell MA. A rapid gas-liquid chromatographic method for the determination of cotinine and nicotine in biological fluids. J Pharm Pharmacol 1990; 42: 450–452.
15. Abrams DB, Follick MJ, Biener L, et al. Saliva cotinine as a measure of smoking status in field settings. Am J Public Health 1987; 77: 846–848.
16. Höld KM, de Boer D, Zuidema J, et al. Evaluation of the Salivette as sampling device for monitoring beta-adrenoceptor blocking drugs in saliva. J Chromatogr B Biomed Appl 1995; 663: 103–110.
17. Etter JF, Perneger TV, Ronchi A. Distributions of smokers by stage: International comparison and association with smoking prevalence. Prev Med 1997; 26: 580–585.

18. Wewers ME, Stillman FA, Hartman AM, et al. Distribution of daily smokers by stage of change: Current Population Survey results. Prev Med 2003; 36: 710–720.
19. Etter JF, Perneger TV. A comparison of cigarette smokers recruited through the Internet or by mail. Int J Epidemiol 2001; 30: 521–525.
20. Haukkala A, Uutela A, Vartiainen E, et al. Depression and smoking cessation: The role of motivation and self-efficacy. Addict Behav 2000; 25: 311–316.
21. Eysenck SBG, Eysenck HJ, Barrett P. A revised version of the psychoticism scale. Person Indiv Diff 1985; 6: 21–29.
22. Pélissolo A, Lepine JP. Normative data and factor structure of the Temperament and Character Inventory (TCI) in the French version. Psychiatr Res 2000; 94: 67–76.
23. Etter JF, Pelissolo A, Pomerleau C, et al. Associations between smoking and heritable temperament traits. Nicotine Tob Res 2003; 5: 401–409.
24. Pomerleau CS, Pomerleau OF, Flessland KA, et al. Relationship of Tridimensional Personality Questionnaire scores and smoking variables in female and male smokers. J Subst Abuse 1992; 4: 143–154.
25. Etter JF, Perneger TV, Ronchi A. Collecting saliva samples by mail. Am J Epidemiol 1998; 147: 141–146.

Address for correspondence: Jean-François Etter, Institute of Social and Preventive Medicine, University of Geneva, CMU, 1 rue Michel-Servet, CH-1211 Geneva 4, Switzerland
Phone: +41-22-379-59-19; Fax: +41-22-379-59-12
E-mail: jean-francois.etter@imsp.unige.ch.